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Performance and in-house validation of a bioassay for the determination of beta1-autoantibodies found in patients with cardiomyopathy

Katrin Wenzel, Sarah Schulze-Rothe, Annekathrin Haberland, Johannes Müller, Gerd Wallukat, Hanna Davideit *

Berlin Cures GmbH, Berlin, Germany

* Corresponding author at: Berlin Cures GmbH, Robert-Rössle Str. 10, 13125 Berlin-Buch, Germany.
E-mail address: davideit@berlincures.de (H. Davideit).

Abstract

Background: Autoantibodies specific for the adrenergic beta1-receptor were identified to be an essential factor for the pathogenesis of dilated cardiomyopathy. For the detection of these autoantibodies, a bioassay was developed and has been used, measuring the positive chronotropic effect on spontaneously beating neonatal rat cardiomyocytes. In order to use this bioassay as an analytical tool to monitor the effectiveness of autoantibody neutralizing therapy in a regulated field, there is a need to assess its analytical performance and validate it according to current guidelines.

Methods: Using standard autoantibody samples, the increased beat rate compared to the basal rate [delta beats/min] was recorded when investigating guideline required assay performance parameters.

Results: The analytical specificity and sensitivity of the bioassay was demonstrated. The limit of detection and positivity cut-off level were determined to be 3.56 and 7.97 delta beats/min, respectively. The coefficient of variation (CV) of all tested single values (four technical replicates each) was ≤15.2%. The CV of precision within each measuring series did not exceed 20%. Furthermore, the sample stability under a variety of different storage conditions was assessed,

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as well as the robustness of the cardiomyocyte preparations, which were both given.

Conclusion: This bioassay fulfilled guideline determined quality requirements and proved to be appropriate for its application in clinical trials.

Keywords: Medicine, Cell biology, Immunology, Cardiology, Health sciences, Biological sciences

1. Introduction

Under physiological conditions, an essential regulator of heart functionality, the adrenergic beta1-receptor, plays a pivotal role in disease development, when target of agonistic acting autoantibodies. A permanent stimulation of the beta1-adrenoceptor, bypassing cell protecting desensitisation processes [1], is a cause [2], [3] and sustainer (for review see [4]) of cell destruction processes, causing cardiomyopathies such as the dilated cardiomyopathy (DCM) (for review see [5]).

The low titre of beta1-adrenoceptor autoantibodies (beta1-AAB) makes an adequate analysis very challenging. So far it has been reported that analytics, based on the exploitation of the autoantibody functionality are superior over tests, using immobilized target moieties especially of peptide origin [6, 7]. Despite intensive efforts, the development of tests which could replace the functional bioassay have often failed. A peptide-based ELISA strategy seems to be questionable [7]. With respect to bioassays, several tests have been developed [8, 9, 10, 11, 12] of which the "classical bioassay" of spontaneously beating neonatal rat cardiomyocytes, developed by Wallukat and Wollenberger as early as 1987 [9], is one of the pioneering tests of the detection of a variety of agonistic acting autoantibodies, including beta1-AAB. This classical bioassay has recently been transferred to a fully automated high throughput level by Joshi-Barr et al. [13]. A FACS assay, based on native beta1-adrenoceptor conformation [14], also proved to be appropriate and comparable to a high degree to the previously mentioned so-called "classical" bioassay of beta1-AAB measurement which is based on the spontaneously beating neonatal rat cardiomyocytes [15]. Other tests which are based on a biological read-out used receptor-mediated changes of contractility of small arterioles (in vitro) as successfully used and described by Li et al. in 2014 [11]. Moreover also cell-based assays using CHO-cells which are transfected with the corresponding receptor of interest such as the human beta1-adrenoceptor using appropriate steps of its signal cascade as read-out, here cAMP production after autoantibody-mediated receptor stimulation, also showed reliable results [12], comparable to the above mentioned arteriole contractility assay [11].

The classical bioassay of spontaneously beating neonatal rat cardiomyocytes has been used before as an exploratory marker for the characterisation of DCM

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patients, positive for beta1-AAB, in this way identifying who would benefit from a therapeutic autoantibody removal [16, 17].

Enabling future clinical testing of autoantibody neutralisation in a regulated field, the classical bioassay was now validated according to the closest current regulations, the "guidance for industry, bioanalytical method validation" dated May 2001 [18] and the European "Guideline on bioanalytical method validation" (came into effect February 1, 2012) [19].

Even though these guidance documents are mainly focused on quantitative assays, still, except for accuracy, all other quality parameters such as precision, selectivity, analytical specificity, reproducibility, and sample stability could have been applied and/or adapted for the validation of this qualitative bioassay. This current study therefore evaluated the analytical performance of the functional bioassay of spontaneously beating neonatal rat cardiomyocytes for beta1-AAB identification.

2. Material and methods

2.1. Material

Goat anti-human-adrenergic beta1-antibody against the 2nd extracellular loop of the beta1-adrenoceptor (ADRB1-AB, cat. no. EB07133), purchased by Everest Biotech Ltd. UK., was used for the preparation of standard material. Bisoprolol hemifumarate (cat. no. B2185) was purchased by Sigma Aldrich (Taufkirchen, Germany).

2.2. Autoantibody sample material

Beta1-AAB containing immunoglobulin-eluate, obtained during the process of regeneration of the IgG-immunoadsorption column within a treatment cycle, was obtained from the Deutsches Herzzentrum Berlin, Berlin, Germany [17], approved by the human Ethics Committee of the Humboldt-University (Berlin, Germany). The IgG-eluate samples (elution with 0.2 M glycine-HCl buffer, pH 2.8) were neutralised immediately using 1 M Tris-buffer pH 7.4 and frozen at −20 °C until further use. Control IgG from the serum of healthy donors was obtained from a blood donation facility (Haema AG, Leipzig Germany) and was shown to be beta1-AAB negative using the bioassay of spontaneously beating rat cardiomyocytes (method description see below). The patients and donors signed an informed consent form.

2.2.1. Immunoglobulin G preparation

To 500 μL of the neutralized eluate fractions or the respective serum control material, 0.33 mL of a saturated ammonium sulphate solution was added slowly

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under gentle agitation. The mixture was incubated for 18 h at 4 °C, followed by centrifugation for 10 min at 3400 × *g*. The resulting pellet was re-suspended in 0.5 mL 0.15 M NaCl, 10 mM phosphate buffer (PBS) pH 7.4 and subsequently mixed with an equal volume of a saturated ammonium sulphate solution and centrifuged again for 10 min at 3400 × *g*. After centrifugation, the pellet was re-suspended in 0.4 mL 0.15 M NaCl, 10 mM PBS and dialysed against 7 × 0.5 L of a buffer consisting of 0.15 M NaCl and 10 mM PBS, pH 7.4 using a VISKING® dialysis tubing membrane (MWCO 14 kD). The final immunoglobulin G (IgG) preparations were aliquoted and stored at −20 °C until testing.

2.2.2. Composition of standard sample material

The analytical assay performance was evaluated using sample material which was similar to clinical samples (sample V1 and V3) and sample material which corresponded to clinical samples (V2, patient identical control material) with the following composition:

Sample V1: IgG preparation of human control serum obtained from a healthy donor spiked with goat anti-human-beta1-adrenoceptor specific antibody (ADRB1-AB). Final amount of ADRB1-AB in the cell culture flask 0.5 μg ADRB1-AB. Sample V2: IgG preparation of sample material from a beta1-AAB-positive patient. Sample V3: IgG preparation of human control serum obtained from a healthy donor spiked with goat anti-human-beta1-adrenoceptor-specific antibody (ADRB1-AB). Final amount of ADRB1-AB in the cell culture flask was 1.0 μg ADRB1-AB.

2.3. Estimation of sample protein concentration

The total concentration of protein in the prepared IgG samples was estimated by measuring the optical density at 280 nm at the Nanodrop 2000c Spectrophotometer (Peqlab, Thermo Scientific, Germany), automatically calculating the amount of IgG using the mass extinction coefficient of 13.7 at 280 nm for a 1% (10 mg/mL) unknown IgG solution.

2.4. Bioassay of spontaneously beating rat cardiomyocytes

2.4.1. Cardiomyocyte preparation

Spontaneously beating cardiomyocytes were prepared from hearts of newborn Wistar rats (1–3 day old, varying number of animals between 20 and 30, (animal experiment license number Y90001/15, Berlin, Germany)). In brief, after decapitation, the hearts of one preparation were immediately removed, transferred into cold (4 °C) PBS without Ca^{2+}, Mg^{2+} (cat. no. L1820, Biochrom, Merck Group, Berlin, Germany) and the ventricles were isolated. Then, the tissue was

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dissected and washed twice in cold PBS. The freshly prepared ventricle pieces were collected in 10 mL PBS, supplemented with 0.2% trypsin and pre-digested for 5 min at 37 °C. The trypsin solution was substituted by 10 mL fresh 0.2% trypsin solution and digested for 15 min at 37 °C. The digestion was stopped by the addition of 5 mL ice-cold heat inactivated calf-serum. The mix was centrifuged at 130 × *g* for 15 min before the resulting pellet was transferred to 5 mL SM20-I medium (cat. no. F9005, Biochrom, Merck Group, Berlin, Germany). The digestion procedure was repeated three times. The cells were counted and adjusted to 2.4×10^6 cells/2 mL which were transferred to a 12.5 cm^2 Falcon cell culture flask (cat. no. 353018, VWR International GmbH, Erlangen, Germany). Cells occurring from one preparation corresponded to one batch.

2.4.2. Procedure of the beta1-autoantibody measurement (bioassay)

Measuring the chronotropic effect of beta1-AAB, the spontaneously beating neonatal rat cardiomyocytes in the 12.5 cm^2 cell culture flask were used, similar to that described before [20]. The basal beating rate (accepted range 100–220 beats/min) of four cardiomyocyte clusters of one flask was recorded using the IonOptix system (including a Carl Zeiss Axio Observer A1 microscope equipped with water bath tempered flask holder in combination with a MyoCam S camera and the recording software IonWizard version 6.5.1. (example in Fig. 1)), or the stop watch-controlled operator counting as described by Wallukat and Wollenberger [9], if stated. The same clusters were measured again after addition of the beta1-AAB-containing sample and incubation for 1 h. The difference from the basal rate was expressed as delta beats per min [Δ beats/min]. In order to exclude interferences from the parallel occurrence of negative chronotropic muscarinic M_2-receptor autoantibodies, the bioassay was performed in the presence of the specific M_2-receptor blocker atropine (1 μM).

A subsequent addition of the specific beta1-adrenoceptor blocker bisoprolol (10 μM) revealed the specificity of the investigated sample when the delta beat rate was reduced to the basal value.

2.4.3. Analytical performance of the bioassay

The analytical performance of the bioassay of spontaneously beating rat cardiomyocytes was assessed creating a protocol according to the FDA released "guidance for industry, bioanalytical method validation" dated May 2001 [18] and the European "Guideline on bioanalytical method validation" (which came into effect February 1, 2012) [19], focusing on the qualitative parameters listed below:

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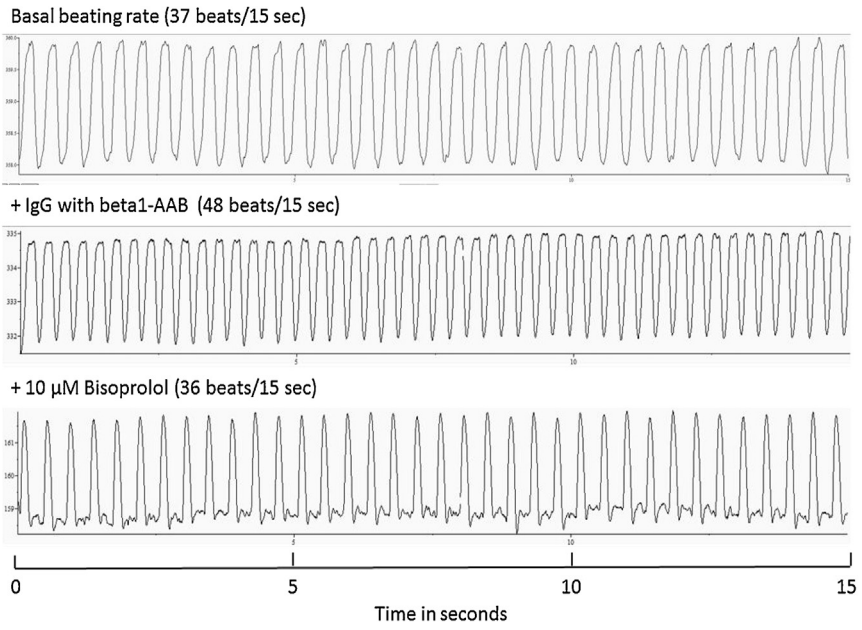

Fig. 1. Example of the measurement of chronotropic effects of beta1-AAB on spontaneously beating neonatal rat cardiomyocytes with the IonOptix system.

2.4.3.1. Limit of detection (LoD)

The limit of detection (LoD) represents the lowest signal of beat rate increase which can reliably be differentiated from background noise. For background noise assessment, four different cell clusters of three different cell culture flasks from one cell batch were measured at three different time points. The LoD was calculated according to:

$$LoD = \text{mean}_{\text{blank}} + 3x(S.D._{\text{blank}})$$

where S.D. stands for the standard deviation of the mean delta beats/min of the single cell culture flasks.

2.4.3.2. Cut-off

The discrimination value between negative and positive samples, the cut-off value, was estimated using raw data of negative samples from the sensitivity evaluation. For this purpose, the data of six beta1-AAB negative samples of varying protein concentrations (reflected varying matrix) were taken and the cut-off was calculated according to:

$$\text{Cut-off} = \text{mean}_{\text{neg}} + 3x(S.D.)$$

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2.4.3.3. Analytical specificity

The analytical sample specificity was assessed using a beta1-adrenoceptor-specific blocking agent bisoprolol. Bisoprolol blocks the beat rate increasing effect of beta1-AAB. Doing this, the beat rate increased effect of beta1-AABs without and with the addition of 10 μM (final concentration) bisoprolol was recorded and compared. For this purpose 2 μL of a 10 mM biosoprolol stock solution in water/ethanol was added to the beta1-AAB containing cell culture medium and incubated for 20 min before the beat rate was registered again.

2.4.3.4. Precision

"*The precision of the analytical method describes the closeness of repeated individual measures of analyte. Precision is expressed as the coefficient of variation (CV)*" [19]. Adopted from the recommendation for quantitative assays, in this qualitative bioassay, a precision of ≤20% at every condition was pre-assigned.

2.4.3.4.1. Intra-assay precision

The intra-assay precision of the bioassay, which is a measure of the variability between five measurements on the same day (five flasks) within the same cell-batch, was assessed using two samples, V1 and V2.

2.4.3.4.2. Intermediate precision

Determining the intermediate precision according to the ICH guideline Q2A and Q2B which "*cover*(s) *the various influences within a laboratory, i.e. conducting analyses on two different (or several) days by different laboratory staff members, with different equipment (if available)*" [21], the *inter-day precision* and also the *inter-cell batch* difference was assessed. For assessment of the *inter-day precision,* aliquots of samples V1 and V2 were analysed on four successive days re-using the same cell culture flasks. This was made possible by decanting the medium of the cell culture flask after the experiments, washing out the autoantibodies using pre-warmed PBS before fresh cell culture medium was added for measurement on the following day.

For the *inter-cell precision* assessment, the beat rate increasing effect of the samples V1 and V2 was analysed exploiting three different cardiomyocyte preparations (three cell batches).

2.4.3.5. Reproducibility

The reproducibility was tested compared to different read-out systems. Here, the automatic beat rate recording of the IonOptix system was compared to the method originally described by [9], a stop-watch controlled operator run beat-rate counting.

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2.4.3.6. Selectivity

According to current guidance documents, selectivity is the ability to detect the analyte of interest in the presence of unrelated matrix compounds (non-specific IgG). Adopted from the recommendation for quantitative assays, in this qualitative bioassay, the selectivity was assessed measuring six independent positive and negative samples of varying protein content covering a range from 0.802 mg/mL to 15.01 mg/mL and 7.629 mg/mL to 10.717 mg/mL, respectively.

2.4.3.7. Sample stability

"Evaluation of stability should be carried out to ensure that every step taken during sample preparation and samples analysis as well as the storage conditions used do not affect the determination of the analyte" [19]. The stability of beta1-AAB functionality was, therefore assessed at different sample storage conditions as listed below:

2.4.3.7.1. Sample freeze-thaw stability

The freeze-thaw stability of beta1-AAB functionality in IgG preparations was assessed at four freeze-thaw cycles using two independent samples, V1 and V2. For logistical reasons, the testing was combined with the *inter-day precision* assessment. The *inter-day precision* data, always generated from material underlying one freeze-thaw cycle only, enabled evaluation of the effect of continuing freeze-thaw cycles on sample stability.

2.4.3.7.2. Sample processing stability

The effect of the sample preparation procedure (see IgG preparation) on the stability and functionality of the beta1-AAB was tested on sample material (V3). The beat rate increasing functionality of ADRB1-AB after the IgG preparation procedure was tested and compared to directly aliquoted ADRB1-antibody.

2.4.3.7.3. Short- and long-term stability

For *short-term stability,* the influence of 30 min incubation at room temperature on sample functionality was evaluated. For this purpose, sample V3 was analysed immediately and data were compared to those generated after 30 min of incubation at room temperature.

Long-term stability assessed the influence of one-month storage at −20 °C and −80 °C. For this purpose, aliquots of sample V3 were stored for one month at the corresponding temperature and were, after thawing, tested for beta1-AAB activity and compared to a freshly prepared V3 sample.

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2.4.3.7.4. Cell preparation stability

Whether a cell culture flask was re-usable after washing out the beta1-AAB for four beta1-AAB measurements on four consecutive days was tested. For this purpose, the beta1-AAB-containing medium of the cell-culture flask was decanted after each measurement, the cells were washed with PBS and fresh medium was added for the new measurement on the following day.

3. Results

3.1. Sample description

Besides samples V1, V2 and V3 (for composition see under Material and methods, samples V1 and V3 are controls, similar to patient sample, and sample V2 is identical to patient sample), six beta1-AAB positive (Pos-1–Pos-6) and six beta1-AAB negative (Neg-1–Neg-6) control samples (IgG preparations) of varying protein concentrations (Table 1) were also included in evaluation of the analytical performance of the bioassay.

3.2. Analytical performance of the bioassay

3.2.1. Limit of detection (LoD)

When assessing the LoD, the mean of the delta beats/min of all measurements amounted to 0.00 delta beats/min ± 1.19 delta beats/min (S.D.). According to the pre-assigned LoD determination (see Material and methods), the LoD amounted to 3.56 delta beats/min and confirmed a historically approximated lab value of 4.0 delta beats/min [22].

3.2.2. Cut-off value

Assessing the *cut-off value*, delta beat rate data from six negative samples as depicted in Table 1 were taken and the cut-off value was calculated according to the equation given in the Material and Methods section (2.4.3.2). It amounted to 7.97 [Δ beats/min] and confirmed a historically approximated lab value of ≤8.0 [Δ beats/min].

3.2.3. Analytical specificity

The *analytical specificity* was assessed by adding the beta1-adrenoceptor specific blocking agent bisoprolol (final concentration 10 μM) to the beta1-AAB-containing sample. A subsequent reduction of the beat rate down or below the cut-off value (point of discrimination between positive and negative samples) was clear proof of analytical specificity. The *analytical specificity* was tested in the beta1-AAB-positive samples Pos1-6 (Table 1). At five of the six tested samples, 10

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Table 1. Raw data[a] for cut-off assessment and testing analytical specificity.

Sample code	Protein conc. [mg/mL]	AAB activity [Δ beats/min]	AAB activity With biso[b] [Δ beats/min]	Mean of AAB [Δ beats/min]	SD of AAB activity [Δ beats/min]	Cut-off[c] [Δ beats/min]
Cut-off assessment						
Neg-1.	7.629	0.00	n.d.			
Neg-2	8.466	3.00	n.d.			
Neg-3	9.047	1.00	n.d.			
Neg-4	8.127	−3.00	n.d.	1.17	2.27	7.97
Neg-5	10.72	2.00	n.d.			
Neg-6	8.134	4.00	n.d.			
Analytical specificity						
Pos-1	0.886	19.0	3.00			
Pos-2	2.185	16.0	−6.00			
Pos-3	15.01	16.0	2.00			
Pos-4	10.07	30.0	4.00	n.d.	n.d.	n.d.
Pos-5	0.802	29.0	9.00			
Pos-6	3.271	18.0	5.00			

[a] Raw data = mean of 4 independent cell cluster [Δ beats/min].

[b] biso = bisoprolol, final conc. 10 μM, incubation for 20 min before measurement.

[c] Mean + 3xSD, n.d. = not determined.

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μM bisoprolol reached the cut-off value of about 8.0 [Δ beats/min], while one sample, Pos-5, showed a clear reduction close to the cut-off value, but still exceeding it by 1 [Δ beat/min].

3.2.4. Precision

3.2.4.1. Intra-assay precision

The intra-assay precision of the bioassay, which is a measure of the variability within one assay was assessed analysing two samples five times on the same day and in the same cell batch. For this purpose, two samples were chosen, V1 (control), and V2 (patient sample). Table 2 shows raw data [Δ beats/min] and the coefficient of variation (CV) for all measurements. The CV for the *intra-assay precision* was, with both sample types, clearly below 20% (V1: 15.2% and V2: 15.6%).

3.2.4.2. Intermediate precision

3.2.4.2.1. Inter-day precision

For the assessment of *inter-day precision,* aliquots of samples V1 and V2 were analysed on four different days by re-using the same cell culture flasks (Table 2), combining the *inter-day* precision and the *cell-re-usability* measurement. This could have been done since the cell culture flask was re-usable after washing out the beta1-AAB for four beta1-AAB measurements on four consecutive days.

The coefficient of variation (CV) for the *intra-day precision* was, with both sample types, clearly below 20% (V1: 15.2% and V2: 12.4%).

3.2.4.2.2. Inter-cell precision

The *Inter-cell precision* assessment was run using 3 different cell preparations (cell batches) and two different samples, V1 and V2 (Table 2). The coefficient of variation (CV) of the measurement between different cell-batches was below 20% with both samples (V1: 5.5% and V2: 12.3%).

3.2.4.3. Reproducibility

The reproducibility was tested by comparing the automatic beat rate recording of the IonOptix system with the original stop-watch controlled operator run beat-rate counting, described by Wallukat and Wollenberger [9] (Table 3). The CV of the positive samples covered 4.0–29.5%. One sample out of six, Pos-1, did not meet the pre-assigned acceptance criteria for CV of 20%. All negative samples were negative (≤8.0 delta beats/min) for both detection systems.

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Table 2. Precision (intra-assay, inter-day, inter-cell batch) assessment: raw data[a] and coefficient of variation (CV) of sample V1 (IgG preparation of ADRB1-AB spiked human control serum) and V2 (IgG preparation of one beta1-AAB containing patient sample).

Sample type	Analysis specifics	Δ beats/min	CV [%]
Intra-assay (Intra-day and Intra cell-batch) precision			
	Analysis No.		
V1	1	10.0	15.2
	2	12.0	
	3	10.0	
	4	14.0	
	5	13.0	
V2	1	26.0	15.6
	2	19.0	
	3	27.0	
	4	20.0	
	5	25.0	
Inter-day precision			
	Analysis day		
V1	1	13.0	15.2
	2	12.0	
	3	11.0	
	4	9.0	
V2	1	29.0	12.4
	2	23.4	
	3	24.0	
	4	22.0	
Inter cell-batch precision			
	Cell-batch		
V1	1	13.0	5.5
	2	11.8	
	3	13.0	
V2	1	23.4	12.4
	2	24.0	
	3	19.0	

[a] Raw data = mean of 4 independent cell cluster [Δ beats/min].

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Table 3. Reproducibility testing: raw data[a] comparing two different detection systems, the IonOptix system versus the originally published visual measuring system.

Sample code	AAB activity IonOptix [Δ beats/min]	AAB activity visual/stop watch [Δ beats/min]	CV [%]
Pos-1	19.0	29.0	29.5
Pos-2.	16.0	17.0	4.3
Pos-3	16.0	17.0	4.3
Pos-4	30.0	23.0	18.7
Pos-5	29.0	27.0	5.1
Pos-6	18.0	17.0	4.0
Neg-1	≤8.0	≤8.0	n.a.
Neg-2	≤8.0	≤8.0	n.a.
Neg-3	≤8.0	≤8.0	n.a.
Neg-4	≤8.0	≤8.0	n.a.
Neg-5	≤8.0	≤8.0	n.a.
Neg-6	≤8.0	≤8.0	n.a.

[a] Raw data = mean of 4 independent cell cluster [Δ beats/min], n.a. = not assessed.

3.2.4.4. Selectivity

The selectivity was assessed by measuring six independent positive and negative samples of varying protein concentrations (Table 1). All negative samples were found to be negative. Delta beats/min were ≤8 [beats/min] in each case and independent of the protein concentration. Unrelated serum proteins (IgG) did not cause false positive results. With respect to the positive samples, all were identified to be positive, independent of the concentration of unrelated IgG.

3.2.4.5. Sample stability

The stability of beta1-AAB functionality was assessed for the following conditions: freeze-thaw stability, sample processing, and sample short- and long-term storage stability.

3.2.4.5.1. Sample freeze-thaw stability

The freeze-thaw stability of beta1-AAB functionality in IgG preparations was assessed for four freeze-thaw cycles using two independent samples, V1 and V2. The acceptance criterion of the four freeze thaw cycles was the reduction of activity compared to fresh samples of less than 35%, which was achieved with both

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Table 4. Freeze-thaw (material V1 and V2) and sample processing stability (V3) at the conditions as indicated in the table. Delta beats/min are the mean of four independent cell cluster of one flask.

Storage condition	Sample type	Freeze/thaw cycle	Δ beats/min[a]	Mean Δ beats/min	Accuracy [%][c]
	V1	0	13.0	11.3	100.0
		1	12.0		92.3
		2	11.0		84.6
		3	09.0		69.2
	V2	0	29.0	24.6	100.0
		1	23.4		80.7
		2	24.0		82.8
		3	22.0		75.9
Sample proc.[b] stability	V3	1	22.0		110.0
	fresh ADRB1	0	20.0		
Short term stability (RT)	V3	0	22.0		100.0
	V3 (30 min)	1	22.0		
Long term stability (−20 °C)	V3	0	22.0		95.5
	V3 (1 month)	1	21.0		
Long term stability (−80 °C)	V3	0	22.0		77.3
	V3 (1 month)	1	17.0		

RT = room temperature.

[a] Δ beats/min = measuring points are mean of four independent cell clusters.

[b] Sample proc. = sample processing.

[c] Accuracy = activity compared to fresh samples ("0") in [%].

samples (Table 4). With sample V1, the maximum loss of activity at the 3rd freeze/thaw cycle was 30.8% while with V2 it was 24.1%, both not exceeding the acceptance criterion.

3.2.4.5.2. Sample processing stability, short- and long-term stability

Testing the influence of the sample processing (IgG preparation) on the stability of the autoantibody, an ADRB1-AB-spiked control sample (V3) was used. Here, the beat rate increasing functionality of ADRB1-AB after the IgG preparation procedure was tested and compared to a freshly aliquoted ADRB1-AB sample. For *short-term stability,* the influence of 30 min incubation at room temperature on sample functionality (V3) was evaluated, while for the *long-term stability,* the influence of one month storage at −20 °C and −80 °C was assessed (Table 4). Autoantibody stability was given for the sample preparation (sample processing) and the tested storage conditions. The ADRB1-AB containing sample V3 showed

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95.5% and 77.3% compared to freshly aliquoted samples when stored for one month at −20 °C or −80 °C, respectively.

4. Discussion

The presented study investigated and describes the analytical performance and in-house validation of the qualitative bioassay of spontaneously beating rat cardiomyocytes for the determination of beta1-AABs found in patients with cardiomyopathy. Spontaneously beating neonatal rat cardiomyocytes are fully appropriate for the detection of human beta1-AAB directed at the first or second extracellular loop of beta1-adrenoceptor since the sequences of the first and second extracellular loops of the human and rat receptors are homolog.

The test, based on the use of living cells, was developed in 1987 [9] and has been in use for almost three decades to date [13, 22]. In order to get an objective view of the robustness and reliability of its performance under routine conditions, especially when applied in a regulated field such as the clinical development of drugs for beta1-AAB neutralisation, it needed, however, to be validated according to current guidelines for bioanalytical method validation. The bioassay was, therefore, in-house validated on the basis of the FDA released "guidance for industry, bioanalytical method validation" (release May 2001) [18] and the European "Guideline on bioanalytical method validation" (which came into effect on February 1, 2012) [19]. Other cell-based assays for autoantibody determination, such as the bioassay measuring thyroid-blocking autoantibodies, have already been successfully validated [23]; this assay, however, is a quantitative assay. A more specific guideline, explicitly aimed at qualitative bioassays, is currently not available.

Under the headline "Method development" (IV. Method development: Chemical Assay)" of the FDA released "guidance for industry, bioanalytical method validation" (release May 2001) [18], there is a recommendation to test the "*fundamental parameters for a bioanalytical method validation*", which would be "*accuracy, precision, selectivity, sensitivity, reproducibility, and stability*". It further describes the single parameter and gives more specific guidance for the execution as well as the pre-assigned tolerated variability limits. Since "accuracy" is tightly connected to quantitative measurements, it had to be adapted in this validation procedure. Accuracy in the qualitative assay narrows down to the verification of the beta1-AAB presence using five determinations not exceeding a CV of 20% a magnitude also recommended for the single precision tests such as intra-batch precision, inter-day precision, and inter-cell precision.

Sample stability is recommended to be tested in order to "*reflect situations likely to be encountered during actual sample handling and analysis*" including "*long-term*

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(frozen at the intended storage temperature) and short-term (bench top, room temperature) storage, and after going through freeze and thaw cycles and the analytical process", testing three aliquots at each condition [18].

Looking at the outcome of the single parameter of the analytical performance testing, it was revealed that the bioassay of spontaneously beating neonatal rat cardiomyocytes is a reliable and robust test with respect to the cell batch to batch reproducibility and with respect to the beta1-AAB sample stability (samples of different origin and under varying storage conditions).

The comprehensive analysis of the performance parameter *intra-assay* and *inter-batch analysis* also revealed satisfying results, showing that the coefficient of variation (CV) was equal to or below 15.2% *for all single measurements (four cell clusters each), which fulfilled guidance recommendations* [18, 19]. The *Analytical specificity* was given in 6 out of six cases with one case still showing a slight remain of the beat rate increase of about 1 Δ beat/min after the addition of bisoprolol. Beta1-AAB independent factor(s), also contributing to the increase of the beat rate in this case were obvious, but did not disturb the beta1-AAB analytic. *The CV for precision analysis and* reproducibility (one exception only) did not exceed 20%. The *Limit of detection* and *cut-off of positivity* were determined to be 3.56 and 7.97 delta beats/min, respectively, revealing values which were already historically described internal lab parameters [22].

5. Conclusion

The bioassay of spontaneously beating neonatal rat cardiomyocytes for the detection of beta1-AAB of patient material fulfilled guidelines determined quality requirements and was shown to be appropriate for routine analytics in a regulated field.

Declarations

Author contribution statement

Katrin Wenzel, Sarah Schulze-Rothe: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Annekathrin Haberland: Analyzed and interpreted the data; Wrote the paper.

Johannes Müller: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Gerd Wallukat: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Hanna Davideit: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Competing interest statement

The authors declare the following conflict of interests: Katrin Wenzel, Sarah Schulze-Rothe, Annekathrin Haberland, Johannes Müller, Gerd Wallukat, and Hanna Davideit are employed by Berlin Cures GmbH. Annekathrin Haberland, Johannes Müller, and Gerd Wallukat are shareholders of the Berlin Cures Holding AG. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject or materials discussed in the manuscript apart from those which are disclosed.

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Additional information

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